

# Blocking of the polyphosphoinositide transmembrane signalling system is a novel and promising approach for AIDS therapy

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**It is now evident that even prolonged and aggressive treatment with combinations of antiretrovirals alone will not lead to HIV eradication and a complete cure. Recent studies indicate that the infection will persist for life, even in patients on effective anti-retroviral therapy. Some of the major reasons for that include the inability of the drugs to stop virus replication completely, their high toxicity and the creation of drug resistance leading to fast viral rebound and transient therapeutic effect. Here we show that blocking of the polyphosphoinositide transmembrane signalling system of HIV target cells by lithium in combination with antiretroviral(s), both requiring obligatory encapsulation in liposomes, overcomes most of the routinely used therapy drawbacks. The extended preclinical and pilot clinical trials evaluating our preparation FTL/AZT/PEBA, based on the above approach, reveal that it is not toxic and may contribute considerably to successful AIDS therapy and the prevention of HIV-promoted malignant transformation.**

## Introduction

### **Current state of HIV/AIDS treatment**

Most, if not all, of the approved drugs currently used for treating HIV/AIDS act by destroying the virus, through the inhibition of its vitally important enzymes such as reverse transcriptase (RT) and/or protease. However, it is now evident that the existing armoury of antiretrovirals and even their triple and/or quadruple combinations, also known as highly active antiretroviral therapy (HAART), will not lead to eradication of HIV infection<sup>1,2,3</sup>. This is mainly due to their inability to affect the DNA form of HIV, high toxicity, insufficient durability of the antiviral effect, low penetration into the HIV reservoir cells and the rapid creation of drug-resistant viral mutants, resulting in transient therapeutic effect and viral rebound. Furthermore, serious metabolic disorders, e.g. lipodystrophy, lactic acidosis, diabetes, osteoporosis and persistent diarrhoea, are observed as a consequence of antiretroviral therapy<sup>4</sup>. Stopping the therapy leads to the re-emergence of HIV, even in patients who had been aviraemic while receiving HAART and intermittent treatment with IL-2<sup>2</sup>. Recently it was reported that, even in patients on effective

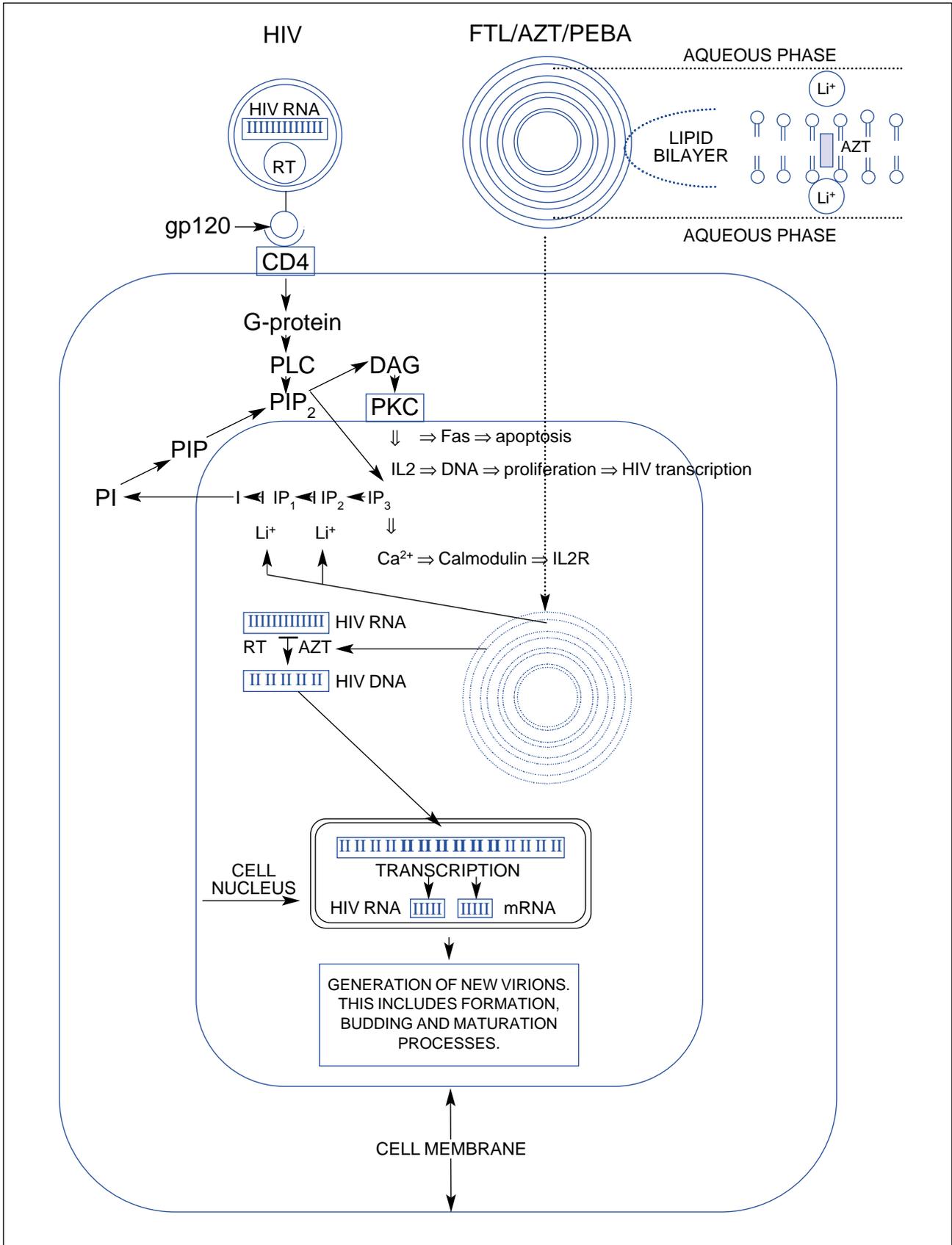
anti-retroviral therapy, silent HIV infection will persist for life and patients need to stay on their medication, possibly for the rest of their lives<sup>5</sup>. The latest findings of the research team led by David Ho provide strong evidence for the inability of current anti-retroviral regimens to suppress viral replication completely<sup>6</sup>.

### **Implication and rationale for blocking transmembrane signalling in HIV/AIDS therapy**

In this paper, we would like to propose a radically new approach for HIV/AIDS treatment which, in contrast to the common strategy of targeting the causative agent exclusively, combines virucidal action with the switching on of the HIV host cell's resources. This is accomplished by blocking the target cell's polyphosphoinositide pathway and, in turn, the generated second messengers, calcium release and the triggering of protein kinase C (PKC)<sup>7,8</sup>, thus rendering the cells refractory to HIV attack (both gp120-stimulated cell activation and/or triggering of apoptosis) as well as blocking viral transcription (**Figure 1**). The crucial role of the above signalling system in cell proliferation, programmed death (apoptosis) and the HIV reproduction process is well documented<sup>7-13</sup>.

We implemented our approach in practice by using lithium as a specific blocker of the polyphosphoinositide pathway<sup>14,15</sup> and 3'-azido-3'-deoxythymidine (AZT, azidothymidine) as an antiretroviral agent, both of them liposome-encapsulated. The latter is an obligatory condition. We have called this

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**Figure 1.** Mechanism of action of FTL/AZT/PEBA (figure not to scale). The active substances AZT and lithium are delivered intracellularly by the liposomes. The antiviral activity of AZT is based on blocking HIV-RT. The effects of lithium are described in the text. It is also reasonable to speculate that lithium ions might possibly add positive charge to the phosphate groups of the cytoplasmic HIV-RT DNA, with consequent inactivation of the molecule. Apparently, further integration of HIV DNA into the cell DNA, with consequent productive and/or latent infection, will thus be avoided. The inherently negative DNA phosphate groups are usually neutralised by cations, which is a strict requirement for DNA functional activity. Lithium-induced positive polarisation will take place in the HIV target cell's cytoplasm only, so that the host cell's nuclear DNA should not be affected. This probably explains the lack of cytotoxicity in our experiments. The role of liposomes, which are not an active drug, is to enhance and focus the therapeutic power of the active substances, such as a concave mirror focuses a light beam without being an energy source by itself.

preparation FTL/AZT/PEBA, which stands for “freeze-thawed liposomes/ azidothymidine/ potent enzyme blocking agent”, the latter being liposome-encapsulated lithium ions, since free lithium ions do not possess the same degree of potency as liposomised ions. The combined mechanism of action and the overall effect of FTL/AZT/PEBA relative to its three constituents are illustrated in **Figure 1**. The contact between the target cell and HIV, and the binding of the CD4 receptor to gp120<sup>16</sup>, results in G-protein-mediated activation of phospholipase C (PLC), an enzyme which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) into two second messengers, 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP<sub>3</sub>)<sup>7,10,12</sup>. The formation of IP<sub>3</sub> induces mobilisation of free Ca<sup>2+</sup> from the intracellular pools with subsequent activation of calmodulin. This results in IL-2 receptor expression<sup>17</sup>. On the other hand, the generation of DAG results in the activation of PKC. The increased concentration of intracellular Ca<sup>2+</sup>, in turn, results in IL-2 production, DNA synthesis, and cell proliferation<sup>7</sup>. In addition to cellular activation and proliferation signals, PKC and calcium-dependent kinases result in the binding of nuclear transcription factors to HIV-LTR, with increased transcription initiation rates<sup>18</sup>.

Lithium is one of the few agents which interferes with the polyphosphoinositide lipid cycle in a selective manner<sup>14,15,17,19</sup>. It inhibits inositol monophosphatase (IMPase), a key enzyme which degrades all inositol monophosphates (Ins 1-P, Ins 3-P and Ins 4-P) to give inositol (I)<sup>15,17,19,20</sup>, as well as inositol polyphosphate 1-phosphomonoesterase, which cleaves Ins (1,4) P<sub>2</sub> to Ins 4-P; it also converts Ins (1,3,4) P<sub>3</sub> to Ins (3,4)P<sub>2</sub><sup>8,15,19</sup>. Lithium-induced inhibition is non-competitive and results in a substantial decrease in inositol and a total increase in monophosphates, Ins (1,4) P<sub>2</sub> and Ins (1,3,4) P<sub>3</sub><sup>8,19</sup>. The resultant depletion of inositol, which is a precursor and the main source for the synthesis of PIP<sub>2</sub>, leads to a lowering of the cellular PIP<sub>2</sub> content to a critical level which, in turn, results in an inability to produce adequate quantities of IP<sub>3</sub> and DAG<sup>15</sup>. Lithium-induced DAG deficiency leads to an inability to activate both DAG-regulated PKC and PKD (protein kinase D, also known as PKC mu). Recently it was shown that PKD is an isoform of the PKC superfamily, which is implicated in a signalling network between different PKCs, operating to amplify and disseminate antigen receptor signals in T-cells, B-cells and mast cells<sup>21</sup>. Apparently, such double blockade of these enzymes, which are vitally important for HIV replication, strengthens the impact on the virus so that, ultimately, it will no longer be able to affect the target cells.

An important role in the further propagation of the HIV replication process is probably also played by myristoylated alanine-rich C kinase substrate (MARCKS), a major PKC substrate, that “has been implicated in cell cycle control”<sup>22,23</sup>. Since the activation of PKC requires DAG<sup>7,15,17,19</sup>, which is decreased by lithium, MARCKS will not be phosphorylated effectively by PKC and HIV replication will be stopped, probably due to the arrest of the virus target cell cycle in G<sub>0</sub><sup>22</sup>.

Recent studies from Stuart McLaughlin’s laboratory revealed that MARCKS strongly binds to membrane

PIP<sub>2</sub><sup>24</sup>. This binding may be frustrated by the lithium-induced PIP<sub>2</sub> decrease. Since the adsorption of MARCKS to plasma membrane was found to be critical for its activity<sup>25</sup>, this may lead to cell cycle dysregulation and the inability of HIV to replicate, as described above.

Moreover, we do not rule out the coexistence of a direct electrostatic interaction between the liposome-delivered intracellular Li<sup>+</sup> and the negatively charged PIP<sub>2</sub>, with subsequent lowering of its hydrolysis by PLC, resulting in second messenger deficiency. Our assumption is based on the electrostatic studies of Stuart McLaughlin and his coworkers<sup>26</sup> on PIP<sub>2</sub>-containing model lipid membranes, which revealed that very low concentrations of different cations (eg. potassium, hydrogen and neomycin) can neutralise (nullify) such negatively-charged lipid membranes.

From the above, it is evident that FTL/AZT/PEBA will stop both proliferation and apoptosis of T-cells caused by HIV, since “they use the same signalling pathway to activate both IL-2 (proliferation) and Fas (apoptosis)”<sup>17</sup>. This is probably the most intriguing feature of our preparation, while being, at the same time, the main perpetrator of the sharp increase in CD4+ T-cells up to normal levels, as seen in our clinical trial. Since lithium blockade takes place long before the bifurcation between proliferation and apoptosis<sup>17</sup> (**Figure 1**), the HIV target cell will thus avoid both virus reproduction with subsequent destruction and/or the triggering of apoptosis, regardless of which of these two “options” it selects. This approach will work equally well in preventing uninfected target cells from being affected by the so-called free gp120 protein circulating in the blood and lymph of people with HIV/AIDS (a process unique to HIV), which may bind to the CD4 receptors of uninfected cells, making them appear infected. This is of utmost importance, since free gp120 leads to the same destructive end effect in uninfected cells as the integral virus has on infected ones<sup>27</sup>. Such cells behave as though they were chronically activated and fail to respond to further activating signals<sup>10,11</sup>. The latter can be explained in the light of severe perturbation of the polyphosphoinositide lipid cycle with consequent accumulation of the second messengers Ins (1,4,5) P<sub>3</sub> and Ins (1,3,4,5) P<sub>4</sub>. This enormous build-up of the above second messengers is caused either by HIV infection or exposure of the cells to free gp120, and is a result of inhibition of the respective enzymes, Ins (1,3,4) P<sub>3</sub> and the Ins (1,3,4,5) P<sub>4</sub>-5’-phosphatases<sup>11</sup>. The additional therapeutic benefit obtained is obvious, since many uninfected cells can thus avoid perishing as a result of contact with free gp120 in the blood, with subsequent apoptosis, misrecognition by natural killer cells and/or creation of a syncytium<sup>27</sup>. Moreover, the probability of their being affected by free gp120 is greater than that of being infected by the integral virus, since fewer than one in 10,000 peripheral blood cells contain HIV RNA, i. e. are infected<sup>7</sup>.

The described abnormalities in inositol phospholipid metabolism caused by HIV and/or its free circulating envelope protein gp120, particularly the excessive accumulation of second messengers, may also contribute to uncontrolled cell division with an end result of

malignant transformation<sup>8,11</sup>. This could explain the appearance of the miscellaneous HIV/AIDS-associated neoplasms which can develop during the course of the disease<sup>27</sup>. Apparently, FTL/AZT/PEBA can provide protection from HIV-induced malignant transformation, by both controlling the phosphoinositide pathway by the above described mechanisms and eliminating the virus.

### **Liposome encapsulation is obligatory**

Unfortunately, for the purposes outlined above, it is not possible to take full advantage of the lithium-induced blockade of the polyphosphoinositide transmembrane-signalling system if lithium is applied in its plain form, as used, for example, in psychiatry for the treatment of manic-depressive bipolar disorders<sup>15,28</sup>. This is due mainly to its high inherent rate of clearance, its low cell membrane penetration ability and resultant low intracellular concentrations, and its high toxicity. In order to overcome the disadvantages mentioned above, we encapsulated lithium (in the form of ultrapure LiCl monohydrate) into liposomes. In accordance with our approach, we also coencapsulated the AZT. This provides a “one-shot” way of simultaneously killing any virus already present and rendering uninfected target cells refractory to it, thus stopping both the further reproduction of HIV and the infection of new cells.

Liposomes, discovered in the mid-1960s by Alec Bangham and colleagues<sup>29</sup>, are artificially produced bilayer lipid membrane vesicles of submicron size, capable of serving as drug carriers<sup>30-32</sup>. The therapeutic use of liposomes was pioneered by Gregory Gregoriadis<sup>33-35</sup>. Liposome drug delivery has the following main advantages over conventional, “free” drugs: enhanced therapeutic efficacy, decreased toxicity, depot effect (sustained release), increased penetration into target and lymphoid cells so that they hit the aetiological agent(s) (e.g. HIV) in both productively infected and reservoir cells simultaneously. When used on small, charged drugs such as Li<sup>+</sup> and AZT, liposome encapsulation acts as a charge shield, strongly facilitating their intracellular delivery and creating constant optimal therapeutic concentration *in situ*. Of course, these properties are highly dependent on the lipid constituents and technology used in producing the liposome formulations.

## **Materials and methods**

### **Preparation of FTL/AZT/PEBA**

We prepared fresh supplies of FTL/AZT/PEBA every week in the form of a stable, isotonic, sterile liposome suspension. Details have been published previously<sup>36</sup>. Briefly, freeze-thawed multilamellar liposomes were subjected to high-pressure argon gas jet homogenisation with subsequent on-line extrusion through 100nm pore size polycarbonate membranes. We used EmulsiFlex-C5 apparatus (Avestin, Canada). In contrast to other investigators<sup>37</sup>, we encapsulated AZT (pharmaceutical quality substance produced by Cipla, India) into the lipid liposome phase instead of the aqueous phase. This approach makes it possible to achieve both a prolonged depot effect and encapsulation of large quantities of AZT. We encapsulated

ultrapure lithium chloride monohydrate (Suprapur; Merck, Germany) into the aqueous liposome phase.

### **Toxicological studies**

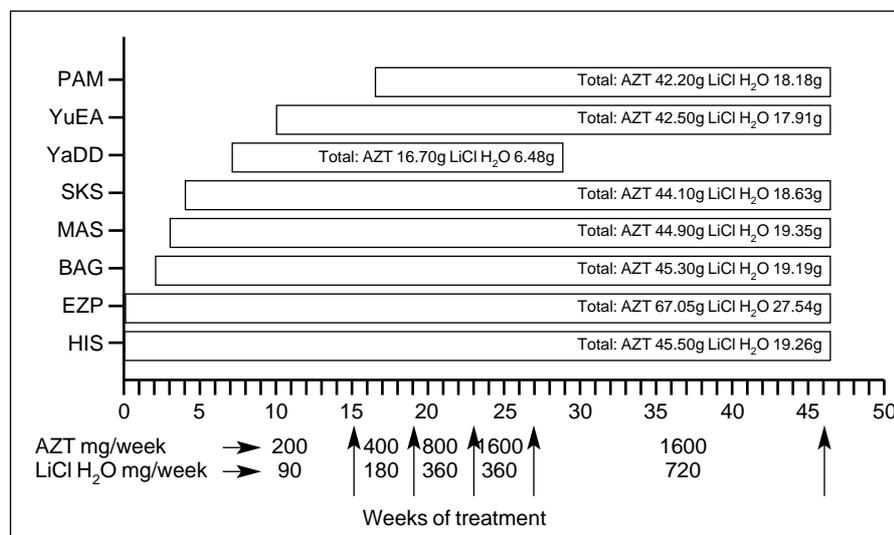
We performed acute (one month) and chronic (seven months, including a one-month wash-out phase) toxicological studies on uninfected experimental mice and rats of both sexes. We used 10 and 100 times, and 1 and 10 times the supposed human dose of the two respective constituents of our preparation for the acute and chronic toxicity trials, respectively. FTL/AZT/PEBA was administered intravenously (i.v.) and intraperitoneally (i.p.) as a single bolus injection at the beginning of the acute study and, in the form of weekly i.v., i.p. and subcutaneous (s.c.) injections during the six-month chronic study. Parallel groups of animals kept under the same conditions were injected with a mixture of free (not liposome-encapsulated) AZT and lithium chloride monohydrate, at the same doses and via the same routes of administration. The respective controls received only a 0.9% pyrogen-free sterile aqueous solution of sodium chloride (Troya Pharm, Bulgaria), again administered via the same parenteral routes.

### **Patient population and measurement of viral load and CD4+ T-cell count**

The pilot trial was carried out on eight male subjects with HIV/AIDS, with a mean age of 33 years (range 22-53), a median initial CD4+ T-cell count of 256 per  $\mu\text{l}$  (range 19-518) and a mean initial viral load (VL) of 93,228 HIV-1 RNA copies/ml (range 2942-328,125). Written informed consent from all of the patients and official permission from the Central Ethics Committee and Ministry of Health were obtained beforehand. At the start of the treatment, five subjects were at the A2, one at the B3 and two at the C3 stage of AIDS. We used the PCR Amplicor HIV-1 Monitor test (Roche Diagnostic Systems, USA) for the quantitative measurement of HIV-1 RNA in plasma and the Simultest IMK-Lymphocyte kit (Becton Dickinson, USA) to obtain flow cytometry measurements of the percentage of CD4+ T-cells. This was then used to compute the absolute CD4+ T-cell count. White blood cell counts and the lymphocyte percentage from an independent differential white cell count were obtained using standard laboratory procedures. All patients tested negative for the presence of *Mycobacterium tuberculosis* by the PCR Amplicor MTB test (Roche Diagnostic Systems, USA). The study was conducted at the “Professor Ivan Kirov” State Hospital for Infectious Diseases in Sofia, Bulgaria.

### **Administering the preparation**

Lacking any prior experience with a similar liposome preparation, we started with doses of 200 mg AZT and 90 mg LiCl monohydrate, administered only once a week, and reached weekly doses of 1600 mg AZT (equivalent to 229 mg/day) and 720 mg LiCl monohydrate (equivalent to 103 mg/day), administered in two divided weekly doses (**Figure 2**). This maximum AZT dose is, however, 2.6 times lower than the dose routinely used in HAART (600 mg/day). We administered the preparation (40 ml) per



**Figure 2.** Individual start- and end-points of the treatment, total FTL/AZT/PEBA dose received (relative to the active substances AZT and LiCl.H<sub>2</sub>O) and the time course of dose escalation. Each division on the vertical axis indicates an individual subject (identified by initials).

rectum using a 50-ml syringe coupled to a catheter. Each patient was given the purgative X-Prep, (Mundipharma, Limburg/Lahn, Germany) one day before administration of the study drug. This route of administration was chosen due to its numerous advantages over the intravenous one: safety (with respect to embolism and sterility), less risk of infection both for patients and personnel, excellent tolerance and compliance, ease of use and very short administration time (3-4 minutes, compared with one hour or more for i.v. infusion). Also, our previous studies on the comparative organ distribution of liposomes following rectal, intravenous and other modes of administration in animals indicated that, 20-24 hours post administration, only rectally administered liposomes can be detected by electron microscopy<sup>36</sup> and fluorimetrically (data not

published) in large quantities in all of the investigated organs, as well as in the brain, providing evidence that the blood-brain barrier had been overcome. Whether this happens after intravenous injection is rather questionable<sup>31</sup>. If one takes into account that the brain is one of the most critical organs and also that it is subjected to both latent and active HIV infection, it becomes evident that rectal liposome drug delivery might be one of the most suitable routes of administration in HIV/AIDS treatment.

### Statistical analysis

We used Prism version 2.01 (Graph Pad Software, USA) both

to construct graphs and to run the following statistical analyses: linear regression, correlation (Pearson  $r$ ), unpaired t-test, non-parametric Mann-Whitney-test, Kolmogorov-Smirnov normality test and two-factor ANOVA<sup>38</sup>. P values are two-tailed.

## Results

### Comparative evaluation of the anti-HIV efficacy of free AZT, FTL/AZT and FTL/AZT/PEBA in vitro

Testing of the above preparations by viral p24 antigen quantification in HIV-infected cell culture supernatants gave the following results: FTL/AZT/PEBA showed 67-fold greater anti HIV efficacy, while FTL/AZT was only 4-5 times more effective, when compared with free

**Table 1:** Clinical laboratory values measured during treatment. Mean values for two treatment periods according to the total dose received (see also Figure 2).

Laboratory Indices	Reference range	Units (SI)	Period 1 (1-23 weeks) (n)	Period 2 (24-46 weeks) (n)	P-value
Erythrocytes	4.6-6.2	10 <sup>12</sup> /l	*3.96 (111)	*4.15 (40)	P < 0.0001
Haemoglobin	140-180	g/l	133.35 (111)	*134.57 (40)	P = 0.5811
Haematocrit	0.40-0.54	l/l	0.39 (111)	0.39 (40)	P = 0.3285
Platelets	140-440	10 <sup>9</sup> /l	179.29 (111)	183.63 (40)	P = 0.0088
Erythrocyte sedimentation rate (Westergren)	<11 (15)	mm/h	20.26 (111)	16.27 (40)	P = 0.0295
Leukocytes	4-10	10 <sup>9</sup> /l	5.00 (111)	*5.61 (40)	P = 0.0012
Protein	58-80	g/l	82.36 (104)	*79.10 (40)	P = 0.0001
SGOT	<22	U/l	19.39 (104)	*17.33 (40)	P = 0.0613
SGPT	<22	U/l	25.59 (104)	*25.73 (40)	P = 0.5580
Alkaline phosphatase	50-170	U/l	46.65 (104)	*53.70 (40)	P < 0.00001
Urea	1.67-8.2	mmol/l	*6.37 (104)	8.62 (40)	P = 0.0032
Creatinine	44.2-133.6	μmol/l	*77.46 (104)	*74.10 (39)	P = 0.003
Glucose	2.78-5.55	mmol/l	4.56 (104)	*4.80 (40)	P = 0.0450
Prothrombin time	80-100	%	87.51 (111)	88.80 (40)	P = 0.0378
Pi	0.77-1.36	mmol/l	*1.41 (104)	1.26 (40)	P < 0.00001
CPK	<80	U/l	46.12 (104)	*51.83 (40)	P < 0.00001
Creatinine clearance	1.3-3.0	ml/s	2.14 (60)	NP	
Bilirubin	3.4-21.00	μmol/l	*17.66 (104)	16.81 (40)	P < 0.00001
Cholesterol	3.36-7.76	mmol/l	3.88 (104)	*4.19 (40)	P < 0.00001

The mean values of the laboratory indices having a Gaussian (normal) distribution in the respective groups are marked with an asterisk (\*). The unpaired t-test was applied where normal distribution applied in both groups. The non-parametric Mann-Whitney test was applied where the distribution of the respective indices was not normal in at least one of the groups. The Kolmogorov-Smirnov test was used to test whether the distribution is normal. NP = not performed.

AZT<sup>39,40</sup>. Results similar to those we achieved with FTL/AZT were obtained using liposomised HIV protease inhibitor<sup>32</sup>. These independent findings lead to the conclusion that liposome encapsulation of anti-retrovirals alone (without lithium) results in similar increases in their antiretroviral efficacy over the “free” drugs *in vitro*, regardless of their different chemical structures and mechanisms of anti-HIV action. When compared to our results with FTL/AZT/PEBA, this clearly demonstrates the crucial role of blocking polyphosphoinositide transmembrane signalling with lithium.

### Toxicological studies

Daily monitoring of the animals' condition, gross pathology, histological and electron microscopic examinations and laboratory haematological and biochemical tests confirmed that there were no detectable short-term and/or long-term toxic changes due to FTL/AZT/PEBA<sup>41</sup>.

### Pilot clinical trial results

No pathological changes in laboratory indices were found; indeed, most of them even showed dose-dependent improvements (Table 1).

The baseline measurements of our patients showed upward VL and downward CD4+ T-cell count linear

regression trends (Figure 3A) typical for progression of untreated HIV/AIDS<sup>27</sup>. Under treatment, these all-patient trends showed a favourable inversion (Figure 3B), indicating the positive therapeutic effect of FTL/AZT/PEBA.

The time-dependent individual dynamics of CD4+ T-cells and VL changes indicated that, under the treatment, 50% of the patients show decreased VL trends (patient HIS 002 has a VL below the detection limit of the PCR) and 63% (five of eight) show upward trends in CD4+ T-cell count (data not shown).

The correlation between CD4+ T-cells and VL for the total individual treatment periods is shown in Figure 4A-H. Six of eight subjects (75%) show an inverse correlation between CD4+ T-cell count and VL (Figure 4A-E, H), i.e. increased CD4+ T-cell count and decreased VL. Both the dynamics and the correlation analysis demonstrate the positive therapeutic effect of the preparation.

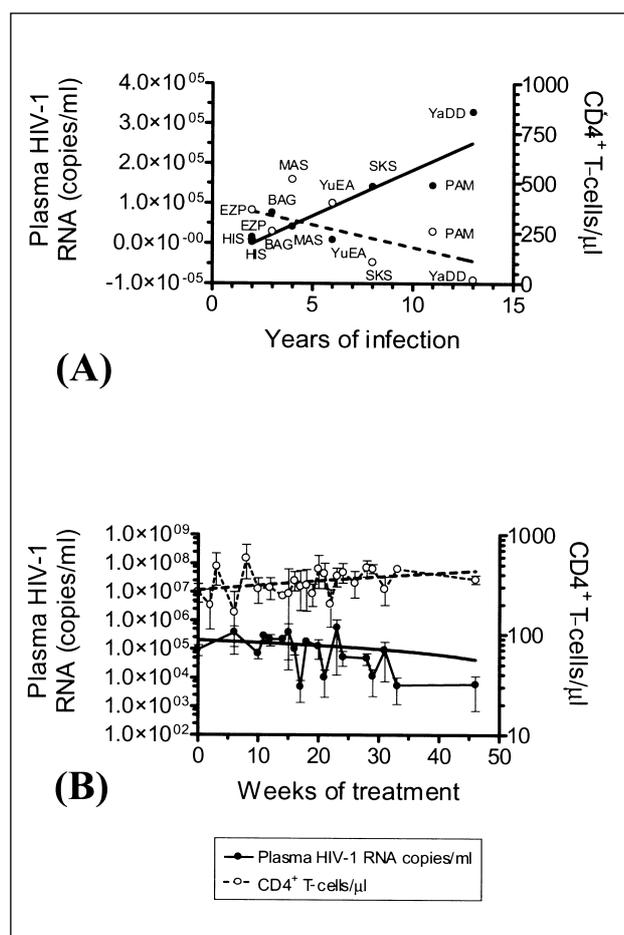
Further evidence of the favourable effect upon the competition between immunity and HIV activity may be seen in the dose-dependent correlation between CD4+ T-cell counts and the VL, measured at different points in time during treatment, corresponding to escalations in the dose of the preparation (Figure 5A-E). An unfavourable correlation exists before the treatment, with an upward VL trend and a downward trend for the CD4+ T-cells ( $r = -0.7552$ ,  $P = 0.0303$ , gradient =  $-516.9 \pm 183.2$ ). This correlation loses its significance during the low-dose treatment period, i.e. after 12 and 18-24 weeks, respectively. However, after 30-36 weeks of treatment, a sizeable inverse correlation is achieved, i.e. a decrease in VL and an increase in CD4+ T-cell count ( $r = -0.9661$ ,  $P = 0.0017$ ), as well as a 33% increase in the gradient ( $-686.3 \pm 91.76$ ) as compared with its pre-treatment value. A further 94% increase in gradient ( $-1003 \pm 266.9$ ), with  $r = -0.8594$  and  $P = 0.0132$ , can be seen after 37-46 weeks of treatment.

The above results are further supported by the two-factor ANOVA performed for the total treatment period, with  $F = 79.17$ ,  $DFn = 7$ ,  $DFd = 131$  and  $P = 0.0001$ . Interaction accounts for approximately 40.75% of the total variance. The effect is considered very highly significant, i.e. the preparation accounts for the changes in CD4+ T-cells and VL.

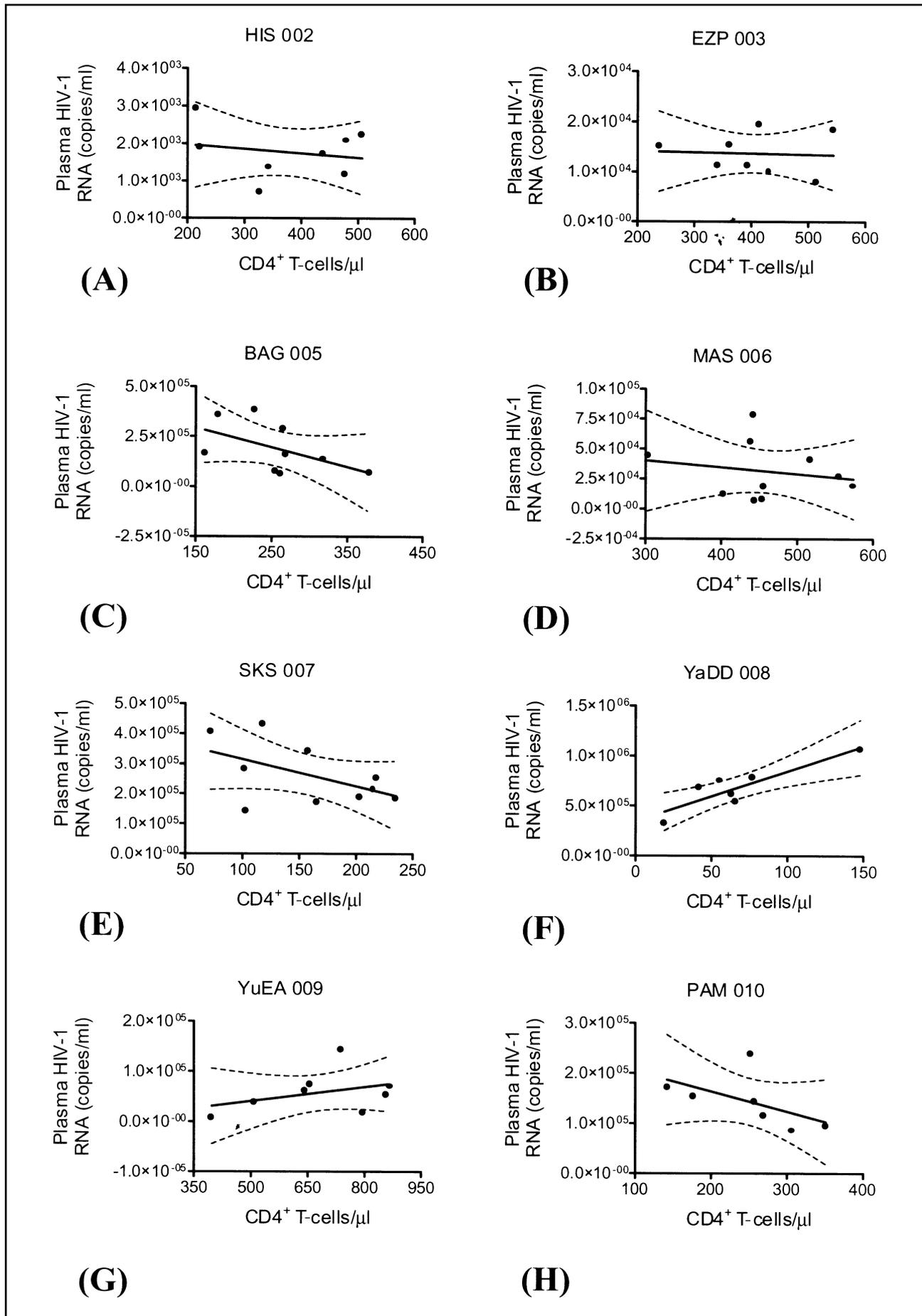
## Discussion

This pilot clinical trial has provided reliable results regarding the safety of the preparation used. It is not toxic: no adverse drug reactions or unwanted side effects were observed. Lack of progression of the HIV infection, with considerable improvements in clinical status and laboratory tests, were also found.

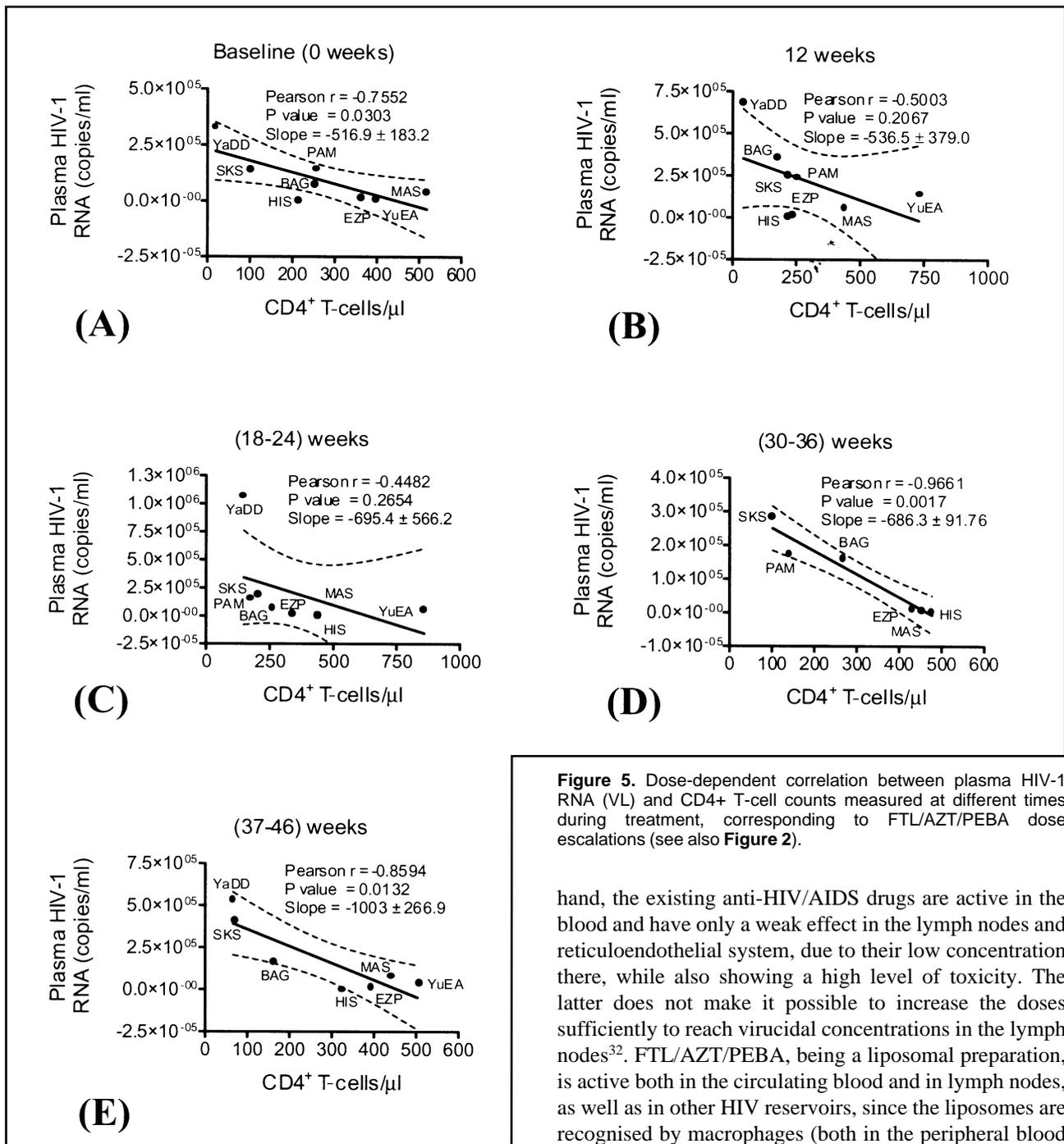
The median total increase in CD4+ T-cells found for the eight subjects is 76 cells/ $\mu$ l. For seven of these subjects (88%), the increase was 119 cells/ $\mu$ ml. The case of one of the subjects (YaDD 008, C3 stage of AIDS) is illustrative of this trend, with an absolute baseline count of 19 CD4+ T-cells rising to 148 CD4+ T-cells in week 23 of treatment, a more than sevenfold increase. Another subject (YuEA 009, A2 stage of AIDS), with an absolute CD4+ T-cell count of 397 at baseline, showed a median



**Figure 3.** Measured values of plasma HIV-1 RNA (VL) and CD4+ T-cells. (A) Before treatment (baseline values). The Y axes are both linear. (B) Mean values during treatment. The inversion of the trends illustrates the positive therapeutic effect of FTL/AZT/PEBA, i.e. the preparation decreases the VL and increases CD4+ T-cell counts. The Y axes are both log10.



**Figure 4.** Correlation between plasma HIV-1 RNA (VL) and CD4<sup>+</sup> T-cells for the individual total treatment periods (see also **Figure 2**). The positive therapeutic effect is illustrated by the inverse correlation between CD4<sup>+</sup> T-cells and VL, ie. increased CD4<sup>+</sup> T-cells and decreased VL in six cases (75%) (A, B, C, D, E, H). (n=8 patients).



**Figure 5.** Dose-dependent correlation between plasma HIV-1 RNA (VL) and CD4+ T-cell counts measured at different times during treatment, corresponding to FTL/AZT/PEBA dose escalations (see also **Figure 2**).

hand, the existing anti-HIV/AIDS drugs are active in the blood and have only a weak effect in the lymph nodes and reticuloendothelial system, due to their low concentration there, while also showing a high level of toxicity. The latter does not make it possible to increase the doses sufficiently to reach virucidal concentrations in the lymph nodes<sup>32</sup>. FTL/AZT/PEBA, being a liposomal preparation, is active both in the circulating blood and in lymph nodes, as well as in other HIV reservoirs, since the liposomes are recognised by macrophages (both in the peripheral blood and in the sanctuary organs) as foreign particles, are consequently phagocytosed and gradually destroyed intracellularly by the lysosome enzymes (lipases), so that the active substances (AZT and lithium) are slowly released *in situ*. From the above, it is evident that our preparation is having to battle against all 100 billion viruses at the same time; naturally, this process requires more time. Besides, the preparation being tested, as mentioned above, greatly increases CD4+ T-cell numbers. This, in turn, increases HIV replication, since they are the target cells where it takes place. This phenomenon is comparable to the predator-pray model of Lotka-Volterra<sup>44</sup>. Indeed, that is exactly what we observed – an increase in CD4+ T-cell count and a temporary increase in VL. After reaching the “turning point”, when the number of the CD4+ T-cells dominates over the already reduced VL, the latter should decrease further. It would appear that

increase of 329 CD4+ T-cells, reaching 867 CD4+ T-cells in week 20 of treatment (normal range 600-1200 cells/ $\mu$ l).

The dynamics of the VL changes under treatment with FTL/AZT/PEBA differ from those obtained with standard preparations. No rapid reduction was found, in contrast with HAART. It is evident that this is due to the gradual dose escalation from a low starting dose and the short period of treatment with the increased doses (**Figure 2**). Moreover, the specific mode of action of the preparation, manifested in its simultaneous antiviral activity in both circulating blood and virus reservoirs, requires longer treatment. It is well known that “about 100 billion new viral particles are produced every day”<sup>42</sup>. From this total body production, only 1% (1 billion) are found in the circulating blood and the remaining 99% are in the HIV reservoirs, mainly in the lymphoid system<sup>43</sup>. On the other

this is what we achieved with some of our patients.

Our study has implications for the development of a more effective strategy for HIV/AIDS treatment. These investigations were performed on HIV-infected cell cultures, experimental animals and subjects suffering from AIDS; now, they need to be extended to larger study populations, using preparations with enhanced anti-HIV efficacy eg. liposome-encapsulated combinations of lithium, AZT, as well as other HIV-RT and/or protease inhibitors. Also, if we take into account the important role of the polyphosphoinositide transmembrane signalling system in cell growth control and tumourigenesis, we have good reason to assume that the general principle of our approach, ie. a liposome-encapsulated combination of lithium and an anti-aetiological/therapeutic agent or agents (eg. suitable anti-tumour drug) might be useful in anti-cancer therapy.

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